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TITLE: Inactivation of FGF Receptors by Targeting Ribozymes Against FGFR mRNAs and Their Effect on FGF Dependent In Vitro and In Vivo Breast Cancer Growth Phenotypes

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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Acquired tamoxifen resistance is common in breast cancer patients with estrogen receptor positive (ER+) tumors. Growth factor signaling can provide ER+ breast cancer cells with alternative growth stimulus to that provided by activation of ER. In order to determine whether an individual FGF receptor (FGFR) or multiple receptors are responsible for conferring an alternate growth signaling pathway, we are using a siRNA targeting strategy to selectively inactivate each of the receptors either singly or in combination. We have designed multiple siRNA against each of the four receptors and screened their ability to reduce target mRNA levels using transient transfection assays. These assays have indicated that at least 3 of 4 individual siRNA against each receptor is able to at least partially reduce target mRNA as determined by quantitative RT-PCR. Using one of these siRNA sequences, we have developed clonal and polyclonal cell lines that stably express siRNA against FGFR3 and shown that these are able to reduce target mRNA up to 90%. We are currently screening additional siRNA against the remaining receptors. These results indicate the feasibility of designing siRNA against the FGFRs and developing stable clones that are able to efficiently knock down target mRNA.

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INTRODUCTION

The majority of patients with breast cancer possess tumors that are positive for the estrogen receptor (1). As a result, these patients are able to benefit from hormonal therapies involving antiestrogenic drugs such as tamoxifen. Unfortunately, these tumors eventually acquire resistance to tamoxifen as well as second line hormonal therapies. The exact cause of this resistance is not known, but interaction of the estrogen receptor with growth factor signaling pathways is thought to play at least a partial role. Previous studies in this lab have shown that inactivation of the Fibroblast Growth Factor Receptors (FGFRs) by over expression of a dominant negative FGFR results in abrogation of tumor cell proliferation under FGF dependent conditions (2). This dominant negative receptor was shown to heterodimerize with at least three of the four receptors and therefore was unable to determine which specific FGF receptor or combination of receptors is responsible for the FGF dependent growth factor signaling. In an effort to determine whether an individual FGF receptor family member or multiple receptors are responsible for conferring an alternate growth signaling pathway, we are utilizing a small interfering RNA (siRNA) targeting strategy to selectively inactivate each of the FGF receptors either singly or in combination.

BODY

During the first year of funding we attempted to observe the effect of ribozyme expression on ML-20 colony formation using transiently transfected ribozymes in anchorage independent colony formation assays. Due to relatively weak growth of ML-20 cells in these assays, we began working with the SW-13 adrenal cortex carcinoma cell line in addition to the ML-20 line. SW-13 cells express FGF cell surface receptors and have shown a more robust growth phenotype in these assays. Due to relatively weak growth of ML-20 cells in these assays, we began working with the SW-13 adrenal cortex carcinoma cell line in addition to the ML-20 line. SW-13 cells express FGF cell surface receptors and have shown a more robust growth phenotype in these assays. SW-13 cells were cotransfected with a blasticidin expression vector and either a CMV-ribozyme expression vector, an inactive ribozyme expression vector, a dominant negative FGFR1 expression vector, or an empty vector control. 24 hours after transfection using calcium phosphate, cells were lifted and 10,000 cells were plated per 35mm dish in a 1mL suspension of 0.36% agar and 10% FBS-IMEM on top of 1mL of 0.6% agar prepared with the same medium. FGF-2 or FGF-4, both of which have been shown to induce robust colony formation in these assays, was added to the top agar mix to a final concentration of 20ng/mL. Heparin sulfate was also included to a final concentration of 50μg/mL. After allowing the agar to solidify at room temperature, cells were incubated at 37°C in a 5% CO2 incubator for 12-14 days. Colonies were counted using Image Pro Plus software (Media Cybernetics, Inc.) If the catalytically active ribozymes were capable of inhibiting FGF dependent growth phenotypes, we expected to see a reduction in colony formation compared to inactive controls and vector controls in media containing FBS + ICI. Results from these experiments indicated that only one ribozyme targeted against FGFR4 appeared to result in a moderate reduction in colony formation by cells transfected with that ribozyme (Figure 1). The remaining ribozymes appeared to have little or no effect on FGF2 or FGF4 induced colony formation in SW-13 cells under anchorage independent conditions.

In order to establish whether or not the ribozymes were actively cleaving target FGFR mRNA, we looked at the ability of the ribozymes to reduce target FGFR mRNA levels using a quantitative RT-PCR approach. For these assays we utilized an improved tetracycline inducible expression vector (pTRE-Ins) designed in our lab (Figure 2). Briefly, ribozymes were placed downstream of a tetracycline response element and upstream of a polyadenylation site. Insulator elements upstream of the TRE and downstream of the polyadenylation site stabilize expression of inserts resulting in reduced silencing and stable expression over extended periods of time. In addition, we have found that this system also exhibits enhanced sensitivity to doxycycline, so lower amounts of the drug can be used to achieve a maximum response. Ribozymes cloned into the pTRE-Ins vector were cotransfected with a zeocin expression vector into a MCF-7 cell line containing the rtTA-S2-M2 reverse tetracycline transactivator (C9). 100mm dishes were plated with $1X10^5$ cells stably expressing pTRE-ribozymes (pTRE-rbz) per dish and colonies were selected for zeocin resistance. After 3-4 weeks, dishes with 500 or more colonies were used to generate polyclonal populations of cells expressing ribozymes in a tetracycline inducible manner.

To determine the ability of inducible ribozymes to inactivate specific FGFR targets, we used a two-step Taqman quantitative RT-PCR assay to observe for reduced target FGFR mRNA levels. Briefly, 7.5 X 10^4 cells were plated per well in 24 well dishes and maintained in IMEM + 5% FBS and incubated at 37°C in a 5% CO2 atmosphere. 24 hours post plating, cells received either fresh media without doxycycline or with doxycycline to a final concentration of 100ng/mL and were incubated for an additional 48 hours. Total RNA was then isolated from triplicate wells of pTRE-ribozyme transfected cells or C9 control cells (RNeasy kit, Qiagen) and cDNA was synthesized using the Advantage™ RT for PCR reverse transcription kit (BD Biosciences) according to manufacturer's suggestions. Optimized primers and probes for each FGFR were designed through Assays by Design (Applied Biosystems). Relative mRNA levels from control cells and pTRE-ribozyme expressing cells, in the presence of or absence of doxycycline, were compared using 18s ribosomal RNA as a housekeeping gene. Results from these experiments indicated that cells expressing induced ribozymes had no significant reduction in target FGFR mRNA levels compared to either C9 control cells, inactive ribozyme control transfected cells, or pTRE-ribozyme transfected cells in the absence of doxycycline (Figure 3).

Based on the results from this and previous assays, we began to consider an alternative approach for inactivating the individual FGFRs. After reviewing several reports from recent literature, we chose a small interfering RNA (siRNA) targeting approach in an attempt to knock down the FGF receptors. To determine the feasibility of using RNA interference (RNAi) to knock down FGFR expression levels, we initially screened one siRNA against each FGFR using quantitative RT-PCR to assess its ability to decrease target mRNA expression. For this initial screen double-stranded and annealed siRNA against individual FGFR, as well as a hrGFP siRNA as a negative control, were obtained from Dharmacon. For design of the siRNAs, full length FGFR sequences were pasted into the Oligoengine web-based siRNA design tool and a potential siRNA target site was selected for each FGFR according to current suggestions for siRNA design. A BLAST search was performed for each siRNA sequence to ensure target specificity. siRNA were transfected into ML-20 cells using Oligofectamine reagent (Invitrogen) according to the manufacturer's protocol to a final siRNA concentration of 5 nM. 24 hours post-transfection, total RNA was isolated using the RNeasy kit (Qiagen), followed by cDNA synthesis using the RT for PCR cDNA synthesis kit (BD). Quantitative RT-PCR analysis was performed as above using the same primer/probe mix. Results from this screen indicated that three of the four siRNA against the FGFRs were at least partially active. mRNA levels in cells transfected with siRNA against FGFRs 1, 3, and 4 were reduced approximately 50, 60, and 25%, respectively, compared to non-transfected ML-20 cells, cells transfected with the hrGFP siRNA, or cells transfected with either siRNA only or transfection reagent only. siRNA versus FGFR 2 appeared to have no effect (Figure 4).

Due to the relative effectiveness of the siRNA sequence designed against FGFR3, we chose to clone this sequence into the pSuper vector (Dharmacon) in order to generate cell lines that stably express active siRNA against FGFR3. The pSuper vector utilizes an

RNA polIII promoter to drive expression of short hairpin RNA (shRNA) sequences that are subsequently processed by intracellular Dicer enzymes into functional siRNA. To clone the FGFR siRNA sequence into the pSuper vector, appropriate sense and antisense oligos (Genosys) containing 5' BglII and 3' HindIII restriction sites were annealed, phosphorylated, and ligated into CIP treated pSuper vector cut with BglII and HindIII restriction enzymes. Each oligo contained complementary siRNA sequences attached by a 9 nucleotide sequence such that when transcribed produce a functional siRNA complementary to the target sequence. Mini-preps were prepared and analyzed by restriction analysis and positive vectors were sequenced to ensure presence and direction of appropriate inserts. ML-20 cells were then plated at various concentrations in 100mm dishes and cotransfected with a FGFR3 siRNA expressing pSuper vector (pSupR3) and a blasticidin resistance vector (pEF6) using Lipofectamine Reagent (Invitrogen). 24 hours post transfection, cells were placed in blasticidin containing media and colonies were selected for 2 to 3 weeks. Polyclonal and clonal populations of cells stably expressing FGFR3 siRNA were generated. For polyclonal populations, dishes containing 500 or more colonies were trypsanized and pooled. Individual colonies were isolated using cloning discs (Sigma) and transferred to 24 well dishes for subsequent expansion. Quantitative RT-PCR analysis, as previously described, was used to screen clonal populations of pSupR3 expressing cells for ability to knockdown FGFR3 mRNA levels. Results indicated that 6 of 6 clonal cell lines were able to reduce receptor mRNA levels by 70 to 90 percent compared to control cells (Figure 5). These results indicate the feasibility of developing clonal cell lines capable of significantly reducing FGFR mRNA expression levels.

Due to the relative inefficiency of siRNAs against receptors 1, 2, and 4, we are currently screening additional siRNA against those receptors. In addition, we are also screening additional siRNA against FGFR3 in an effort to achieve higher siRNA efficiency against that receptor. For this purpose, 5 different target sites were chosen for each receptor and new siRNA were constructed using the Ambion Silencer siRNA construction kit against these target sites. In addition to the 5 new target sites, the previously screened Dharmacon sequences were also constructed using the same kit in an effort to determine the relative efficiency of chemically synthesized siRNA versus siRNA constructed by the kit. These siRNAs will be screened by quantitative RT-PCR as described previously.

Key Research Accomplishments

- Shown that transient expression of ribozymes directed against individual FGF receptors is not effective at reducing SW-13 colony formation as determined by anchorage independent colony formation assays
- Shown that doxycycline inducible ribozymes are ineffective at reducing target FGFR mRNA as determined by quantitative RT-PCR analysis
- Screened individual chemically synthesized siRNA against each of the four FGFRs and shown reduction of FGFRs 1, 2, and 3
- Designed and constructed 6 siRNA against each of the four FGFRs, plus one negative control siRNA (hrGFP)
- Shown that siRNA designed against FGFR3 are able to knock down receptor mRNA levels 80% as determined by quantitative RT-PCR analysis using transient transfections
- Inserted the active siRNA sequence against FGFR3 into the pSuper vector and isolated clones that are able to stably knock down FGFR3 mRNA levels up to 90% as determined by quantitative RT-PCR analysis
- Presented data at the Department of Defense 2002 Era of Hope convention

Reportable outcomes

Norman Estes, Jaideep Thottassery, and Francis G. Kern. Inactivation of FGF Receptors by Targeting Ribozymes against FGFR mRNAs and Their Effect on FGF Dependent *in vitro* and *in vivo* Breast Cancer Growth Phenotypes. Department of Defense Breast Cancer Research Program Meeting. September 25-28, 2002, Orlando Florida, P36-3.

Conclusions

At the end of the second year of this study, we have determined that multiple ribozymes designed against each FGFR are mostly ineffective at reducing target mRNA levels as determined by quantitative RT-PCR analysis. In addition, these ribozymes appeared to have little effect on FGF dependent growth phenotypes as determined by anchorage independent colony formation assays. We are now using a siRNA targeting approach in an attempt to selectively inactive the FGFRs. We have already designed and tested individual chemically synthesized siRNA against each receptor. Using quantitative RT-PCR, we have determined that 3 of the 4 siRNA are able to at least partially reduce target mRNA levels. Using one o these siRNA we are able to effectively reduce FGFR3 mRNA levels by 80%. Using this siRNA sequence, we have developed clonal and polyclonal populations of cells that stably express siRNA against FGFR3. Quantitative RT-PCR analysis of these cells indicate a 90% reduction in FGFR3 mRNA levels. In addition to the previously screened siRNA, we have also developed additional siRNA against each receptor and are currently screening these using quantitative RT-PCR.

References

1. Kern, F., Role of Angiogenesis in the Transition to Hormone Independence and Acquisition of the Metastatic Phenotype. Contemporary Endocrinology: Endocrinology of Breast Cancer, 1998.

2. Zhang, L., Kharbanda, S., Hanfelt, J., and Kern, F.G. Both autocrine and paracrine effects of transfected acidic fibroblast growth factor are involved in the estrogen-independent and antiestrogen-resistant growth of MCF-7 breast cancer cells. Cancer Research, 58:352-361. 1998.

Appendix

Figures and Legends

Abbreviations

CCS	charcoal stripped fetal calf serum
EDTA	ethylenediamine-tetraacetic acid
ER	estrogen receptor
ER+	estrogen receptor positive
ERK1/2	extracellular signal regulated kinase 1/2
FGF	fibroblastic growth factor
FGFR	fibroblastic growth factor receptor
FBS	fetal bovine serum
GCG	genetics computer group
GFP	green fluorescent protein
hrGFP	humanized Renilla green fluorescent protein
IMEM	Improved Minimal Essential Medium
MAPK	mitogen activated protein kinase
RNA	ribonucleic acid
RNAi	RNA interference
shRNA	short hairpin RNA
siRNA	small interfering RNA
mRNA	messenger ribonucleic acid

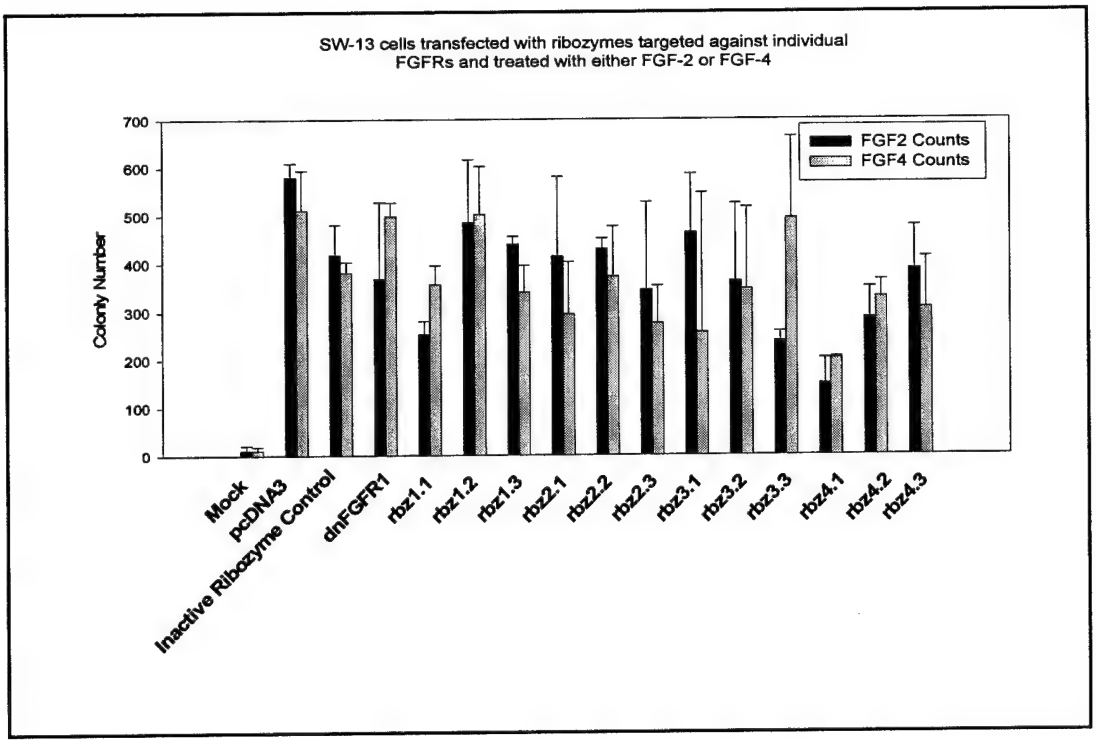

Figure 1 Expression of ribozymes against individual FGFRs were mostly unable to reduce colony formation of SW-13 adrenal cortex carcinoma cells stimulated with either FGF2 or FGF4 in soft agar colony forming assays compared to various controls. Cells were transfected with ribozyme expression vectors or control vectors. 24 hours after transfection, cells were plated in a 0.36% layer of agar containing 5% FBS and conditions atop a bottom layer of 0.6% agarose containing the same media without conditions. Cells were plated in triplicate in 35mm dishes at 10,000 cells per dish and incubated for 12-14 days before colonies were counted using Image Pro Plus software (Media Cybernetics, Inc.)

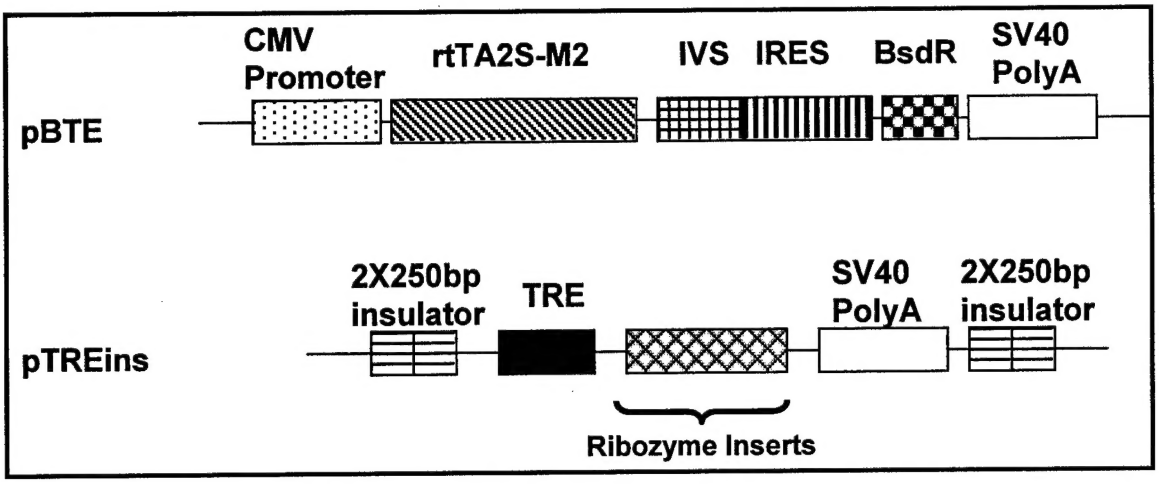

Figure 2 pTRE-Insulator constructs previously developed in our lab and used in construction of inducible ribozyme expression vectors. Ribozyme sequences were placed downstream of a tetracycline response element and upstream of a polyadenylation site. Insulator elements upstream of the TRE and downstream of the polyadenylation site stabilize expression of inserts resulting in reduced silencing and stable expression over extended periods of time. In addition, an internal ribosomal entry site allows for expression of a blasticidin resistance marker. ML-20 cells were transfected with the pBTE construct and stable clones were selected. Stables clones were isolated and subsequently verified to stably express the rtTA2S-Ms reverse tetracycline transactivator. These cells (C9) were subsequently transfected with the pTREins vector containing ribozyme sequence inserts and stable cells lines were developed that expressed ribozymes against specific target FGFRs.

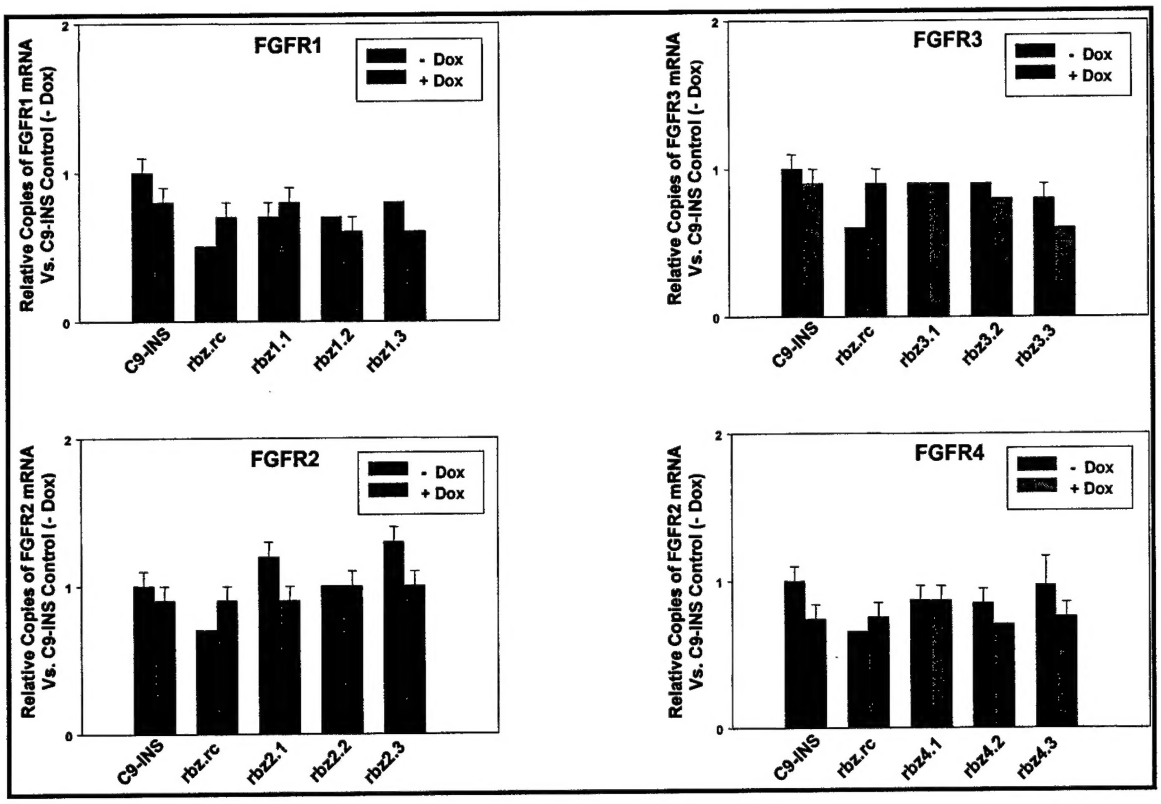

Figure 3 Quantitative RT-PCR analysis of C9 cells stably transfected with doxycycline-inducible ribozyme expression vectors against the indicated FGFR in either the presence or absence of doxycycline. Induction of ribozymes with 100ng/mL doxycycline does not significantly reduce FGFR mRNA levels below that of control cells or non-induced cells. mRNA levels are expressed relative to the non-induced C9 mRNA levels which are arbitrarily set to 1.

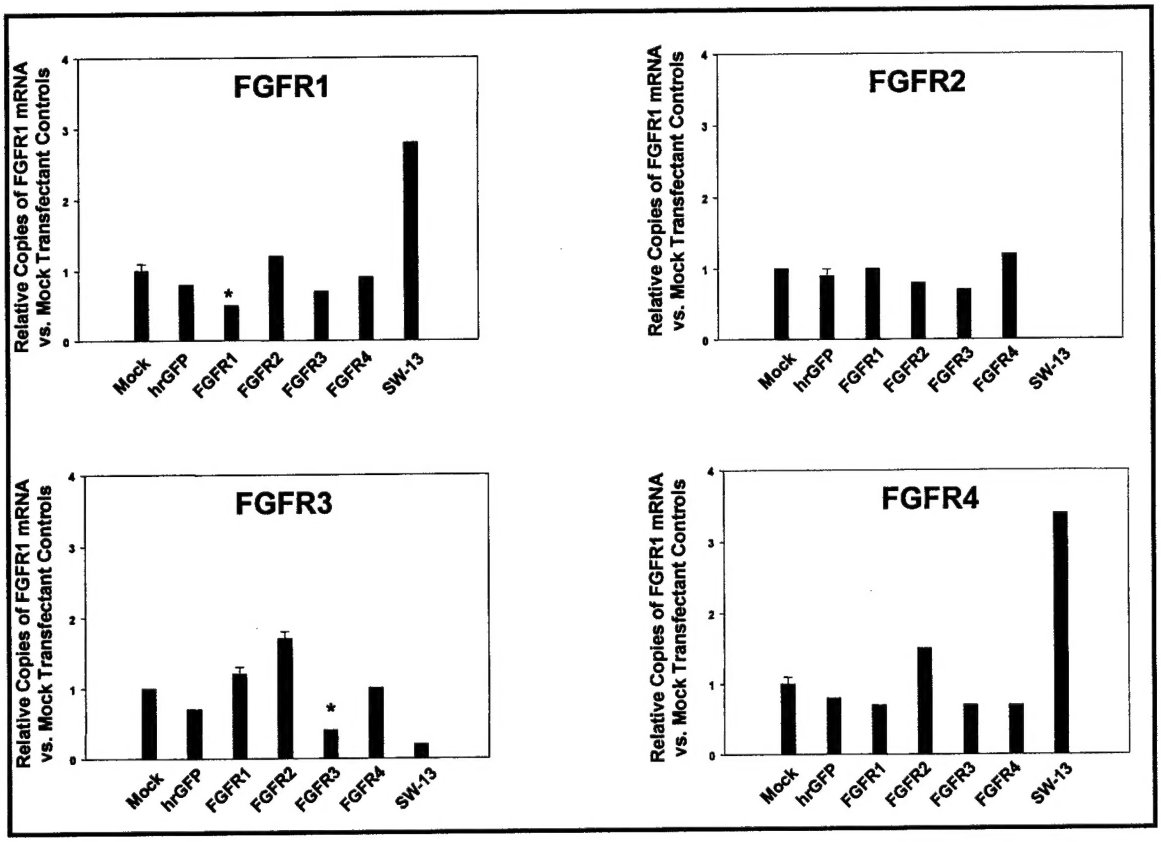

Figure 4 Quantitative RT-PCR analysis of ML-20 cells transiently transfected with siRNA against individual FGFRs. Cells were transfected with single siRNA against either hrGFP, used as a negative control, or against a specific FGFR and then probed for the indicated receptor. Reduction in target mRNA was compared to mock transfected cells whose mRNA values were arbitrarily set to 1. The asterisks indicate significant difference as determined by a t-test with $p < 0.05$.

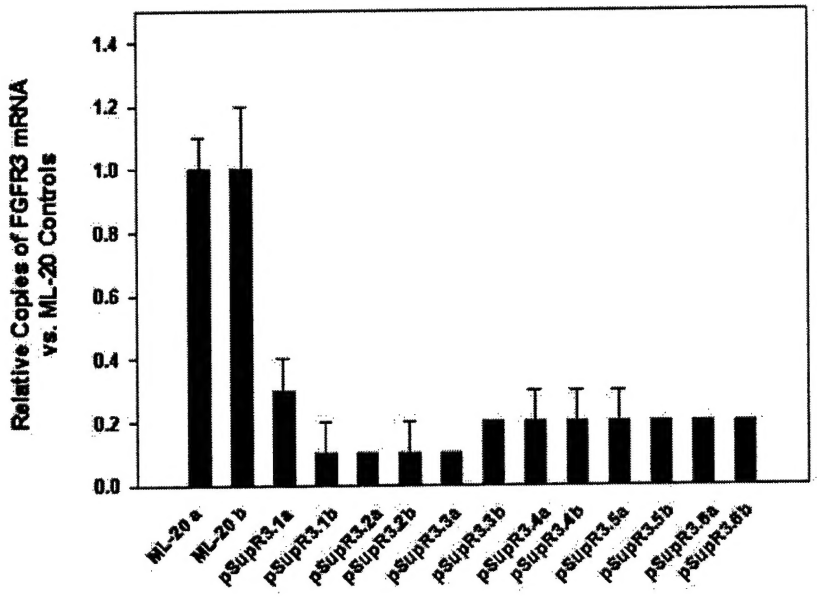

Figure 5 ML-20 cells stably expressing a siRNA targeted against FGFR3 (pSupR3) are able to effectively know down FGFR3 mRNA levels 70-90% compared to parental ML-20 cell line.